[CANCER RESEARCH 63, 2388–2392, May 15, 2003]

Identification of Mucin-depleted Foci in the Unsectioned Colon of Azoxymethane-treated Rats: Correlation with Carcinogenesis[1]

Giovanna Caderni,[2] Angelo Pietro Femia, Augusto Giannini, Alessandro Favuzza, Cristina Luceri, Maddalena Salvadori, and Piero Dolara

Department of Pharmacology, University of Florence, 50139 Florence, Italy [G. C., A. P. F., A. F., C. L., M. S., P. D.], and Department of Pathology, S. M. Annunziata Hospital, Florence [A. G.], Italy

ABSTRACT

We tested the association between aberrant crypt foci (ACF) and tumor induction by feeding azoxymethane-induced rats (15 mg/kg × 2, s.c.) with synbiotics (Raftilose Synergy 1, a derivative of inulin, 10% of the diet, along with lactobacilli and bifidobacteria). After 16 weeks of feeding, synbiotics significantly increased ACF multiplicity. On the contrary, after 32 weeks, synbiotics significantly decreased intestinal tumors. When the same unsectioned colon used for ACF determination was stained with high-iron diamine Alcian blue, foci of crypts with scarce or absent mucins were identified. We defined these lesions as mucin-depleted foci (MDF), and they were visible in all azoxymethane-treated rats and correlated with tumor induction (MDF/colon: 8.2 ± 0.9 and 3.8 ± 0.9 in controls and synbiotic-fed rats, respectively, $P < 0.01$; crypts/MDF: 12.2 ± 2 and 6.4 ± 1 in controls and synbiotic-fed rats, respectively, $P < 0.05$, means ± SE, $n = 7$). There were fewer MDF/colon than ACF, and they were histologically more dysplastic than mucinous lesions identified as ACF in high-iron diamine Alcian blue-stained colon. In conclusion, MDF may be premalignant lesions that predict colon carcinogenesis.

INTRODUCTION

Colon carcinogens such as AOM[3] or 1,2-dimethylhydrazine induce colon cancer in rodents through a multistep process characterized by the sequential formation of histopathological lesions similar to those observed in spontaneous carcinogenesis in humans (1). As part of this process, ACF have been identified as preneoplastic lesions visible in the unbedded colon of rodents as early as 2 weeks after carcinogen administration (2, 3). ACF have also been identified in humans (4, 5), are easy to quantify in the entire colon, and have a preneoplastic nature (6–10). Therefore, ACF determination has been widely used as a short-term test for predicting colon carcinogenesis (11), although some studies reported conflicting results on the association between ACF and tumor development (12, 13). In fact, in humans and rodents, ACF have variable levels of dysplasia, and it has been suggested that only a few dysplastic ACF will develop into cancers (7, 8, 14). Recently, "β-catenin accumulated crypts" have been described as possible premalignant lesions in AOM-treated rodents (15). Although these lesions and dysplastic ACF may represent true preneoplastic lesions, their identification in the unsectioned colon is problematic because there is no practical way to look at the entire colon of a rat histologically.

Given these considerations, we were interested in correlating ACF and tumors in a study in which AOM-treated rats were treated with synbiotics, compounds with potential chemopreventive activity (16), comprising a prebiotic, Raftilose Synergy 1 (a derivative of inulin), and the probiotics, lactobacilli and bifidobacteria. Moreover, in the unsectioned colon of the treated animals, we searched for crypt foci with altered mucin production, one of the most prominent features of dysplasia in the colon (17), hoping to find alternative biomarkers for cancer. This led us to the identification of MDF, which may be precursor lesions of colon tumors.

MATERIALS AND METHODS

AOM was purchased from Sigma (Milan, Italy). Raftilose Synergy 1, a derivative of inulin, was provided by Orafti (Tienen, Belgium). *Lactobacillus GG, L. delbrueckii* subsp. rhamnosus, and *Bifidobacterium lactis* Bb12 were provided by Valio (Helsinki, Finland) and purchased from Chr. Hansen (Horsholm, Denmark), respectively.

Animals and Treatments. We used 4–5-week-old, male F344 rats (Nossan, Correzzana, Milan, Italy). The animals were housed according to the European Union Regulations on the Care and Use of Laboratory Animals, as reported previously (18). Rats ($n = 92$) were randomly allocated to two groups with the following diets. The control group ($n = 46$) was fed a high-fat diet, based on the AIN76 diet (16), modified to contain a high amount of fat [230 g/kg corn oil (w/w)], a low level of cellulose [20 g/kg (w/w)] and maltodextrins [100 g/kg (w/w)], and sucrose [360 g/kg (w/w)] as sources of carbohydrates. The synbiotic group ($n = 46$) was fed the same diet as controls, but maltodextrins were replaced by 100 g/kg (w/w) Raftilose Synergy 1; this diet also contained *Lactobacillus GG, L. delbrueckii* subsp. rhamnosus, and *Bifidobacterium lactis* Bb12 strains (5×10^8 colony-forming units of each strain/g diet).

Ten days after beginning the feeding of the experimental diets, rats were injected with AOM (1 week apart; 15 mg/kg × 2, s.c). In each dietary group, some animals were treated with saline instead of AOM (4 animals in the control group and 5 animals in the synbiotic group).

Either 7 or 15 weeks after the first AOM injection, two groups of seven rats (control and synbiotic groups) were sacrificed by CO_2 inhalation, and ACF were determined according to Bird (2). The colons were coded and scored independently by two observers. The correlation coefficient between the scores of the two observers on a set of 60 colon samples was 0.78 ($P < 0.0001$) for the number of ACF/colon and 0.87 ($P < 0.0001$) for the multiplicity of the ACF (ACs/ACF).

Thirty-one weeks after the first AOM injection, tumors were determined in the two experimental groups (synbiotic and control groups, 28 animals/group) using methods previously described in detail (16, 18).

Determination of Mucin Production in the Unbedded Colon and Identification of MDF. Mucin production was analyzed by restaining with the HID-AB procedure formalin-fixed colons that were previously stained with methylene blue to visualize ACF, as described previously (18). The HID-AB-stained, unbedded colons were then scored at the microscope (×40 magnification), mucosa side up. MDF were identified as focal lesions by the following criteria: (*a*) absence or very small production of mucins; (*b*) distortion of the opening of the lumen compared with normal surrounding crypts; (*c*) elevation of the lesion above the surface of the colon; and (*d*) multiplicity (*i.e.*, the number of crypts forming each focus) higher than 3 crypts. To be defined as MDF, a focus had to fulfill the first criterion (absence or very low production of mucins) and at least two of the other criteria listed above. The colons were coded and scored independently by two observers. The correlation coefficient between the scores of the two observers on a set of 14 colon samples was 0.86 ($P < 0.001$) for the number of MDF/colon and 0.91 ($P < 0.001$) for the multiplicity of MDF (number of crypts/focus).

Dissection of MDF and ACF and Evaluation of Dysplasia. MDF and ACF were identified at the microscope as described above, marked with permanent ink (The Davidson Marking System; Bradley Products, Blooming-

Received 7/29/02; accepted 3/13/03.

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[1] Supported by EU Projects QLKI-1999-00346 and QLRT 1999-00505 and by Ministero Ricerca Scientifica Tecnologica (MURST), Italy.

[2] To whom requests for reprints should be addressed, to e-mail: giovanna.caderni@unifi.it.

[3] The abbreviations used are: AOM, azoxymethane; AC, aberrant crypt; ACF, aberrant crypt foci; MDF, mucin-depleted foci; HID-AB, high iron diamine–alcian blue.

ton, MN) during microscopic observation, dissected, and then embedded in paraffin in such a way that the crypts could be sectioned longitudinally. Sections (4 μm) were stained with H&E. Dysplasia was evaluated in at least 2 sections/focus (approximately 100 μm apart) by a pathologist unaware of the topographical classification of the lesion by studying the different parameters that characterize dysplasia in pathology (17). An arbitrary score was given to each parameter with the following criteria: (*a*) nuclear stratification (0, none; 1, mild; 2, severe); (*b*) loss of nuclear polarity (0, none; 1, mild; 2, severe); (*c*) increase of nuclear:cytoplasmic ratio (0, 0–25%; 1, 25–50%; 2, >50%); (*d*) irregularity in the nuclear outline (0, none; 1, mild; 2, severe); (*e*) nuclear crowding, *i.e.*, increase of basally located and overlapping nuclei (0, none; 1, mild; 2, severe); (*f*) number of mitoses (0, none; 1, 1–3 mitoses/lesion; 2, >3 mitoses/lesion); (*g*) mucin depletion (0, absence; 1, mild; 2, severe); and (*h*) structural abnormality of the crypts (0, absence; 1, mild; 2, severe). When different sections of the same specimen had a different score for the same parameter, we chose the highest value. The total dysplasia score was the sum of the scores relative to each parameter considered for each sample.

RESULTS

The results of the carcinogenesis experiment, 31 weeks after AOM, demonstrated that rats treated with prebiotics and probiotics (synbiotics) had a significantly lower number of intestinal tumors (Fig. 1*A*) and a lower incidence of intestinal tumors (Fig. 1*B*) when compared with controls. On the contrary, in a parallel feeding experiment, rats in control and synbiotic-fed groups, sacrificed 15 weeks after AOM, had a similar number of ACF/colon (Fig. 1*C*; Table 1), whereas the multiplicity of ACF was even higher in the group treated with the synbiotics than in controls (Fig. 1*D*; Table 1). Similar results were obtained in rats sacrificed 7 weeks after AOM (Table 1).

The same methylene blue-stained unbedded colons that served for the evaluation of ACF as reported above were restained with HID-AB to highlight mucin production (18). We reported previously (18) that ACF can be easily observed and counted even in unsectioned colons stained with HID-AB. Accordingly, in the present study, we found that the number and multiplicity of ACF evaluated in 14 samples of distal colon stained with methylene blue were significantly correlated to the number and multiplicity of mucinous lesions identified as ACF evaluated in the same 14 samples restained with HID-AB after methylene blue (mean ± SE values of ACF/distal colon were 92.2 ± 4.9 and 86.1 ± 4.4 in methylene blue-stained and HID-AB-stained colons, respectively; correlation coefficient between the two scores = 0.53, $P < 0.05$; mean ± SE values of ACs/ACF were 2.40 ± 0.10 and 2.39 ± 0.12 in methylene blue-stained and HID-AB-stained colons, respectively; correlation coefficient between the two scores = 0.93, $P < 0.001$).

In the whole colon stained with HID-AB, besides mucinous lesions identified as ACF (Fig. 2*A*), it was possible to identify foci of crypts characterized by depletion of mucins (Fig. 2, *B* and *C*), which we defined as MDF. The characteristic trait of MDF was the absence or very low production of mucins. This trait has been reported as a hallmark of dysplasia (9, 17), and, accordingly, we assumed that MDF might be dysplastic lesions. MDF were easily detected in AOM-treated rats, whereas saline-treated rats did not exhibit such lesions.

To test whether MDF were related to carcinogenesis, we determined the number of MDF and their multiplicity (number of crypts/MDF) in controls and synbiotic-treated rats. Seven weeks after AOM, MDF were already visible in the colon, and the number and multiplicity of MDF were similar in the two groups (Table 1). On the contrary, in rats sacrificed 15 weeks after AOM, the number of MDF was significantly lower in the rats treated with synbiotics (Fig. 1*E*; Table 1). Moreover, 15 weeks after AOM, the multiplicity of MDF in rats fed synbiotics was significantly lower than that in controls (Fig. 1*F* and Table 1). Similar to what was observed for the ACF (Table 1), the multiplicity of MDF was higher 15 weeks after AOM than after 7 weeks (Table 1), a phenomenon particularly evident in rats fed the control diet. Moreover, MDF were more numerous in the rats fed the control diet that were sacrificed 15 weeks after AOM than in rats sacrificed after 7 weeks (Table 1); this phenomenon did not occur in synbiotic-fed rats.

The MDF and mucinous lesions identified as ACF in HID-AB-stained colons were then sectioned, and their dysplasia was assessed with a semiquantitative score in longitudinal sections, as described in "Materials and Methods." Examples of histological sections of MDF

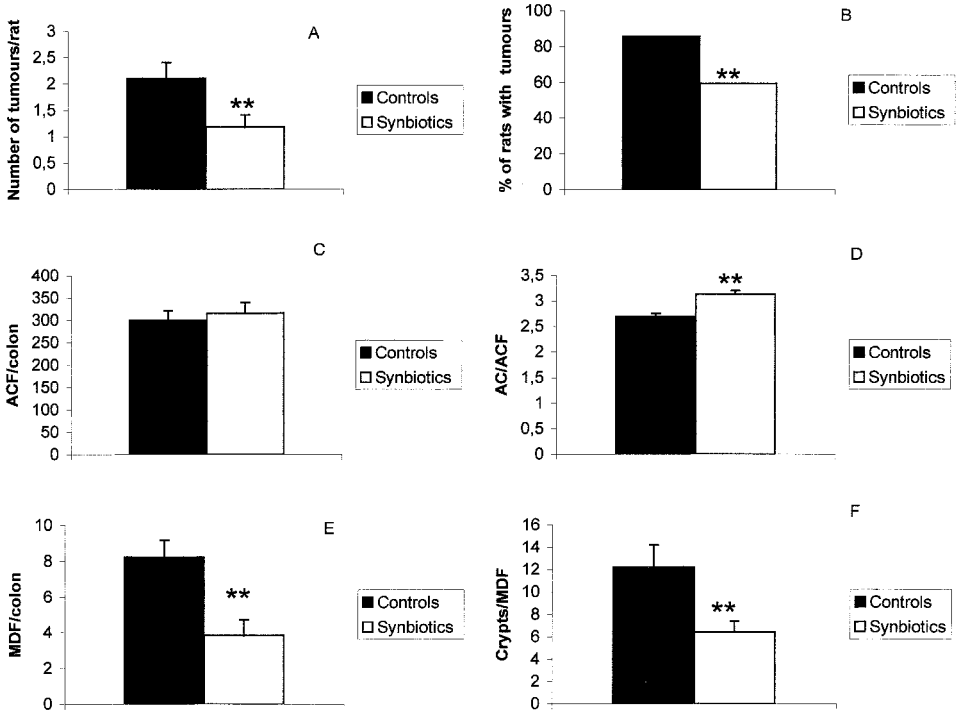

Fig. 1. *A*, number of intestinal tumors/rat in animals fed a control diet or a diet containing synbiotics; 31 weeks after the first AOM treatment. *B*, incidence of intestinal tumors (rats with tumors in each group × 100) in rats fed a control diet or a diet containing synbiotics; 31 weeks after the first AOM treatment. *C*, number of ACF/colon (evaluated in methylene blue-stained colon) in rats fed a control diet or a diet containing synbiotics; 15 weeks after the first AOM treatment. *D*, number of ACs/ACF in rats fed a control diet or a diet containing synbiotics; 15 weeks after the first AOM treatment. *E*, number of MDF/colon (evaluated in HID-AB-stained colon) in rats fed a control diet or a diet containing synbiotics; 15 weeks after the first AOM treatment. *F*, number of crypts/MDF in rats fed a control diet or a diet containing synbiotics; 15 weeks after the first AOM treatment. **, significantly different when compared with controls, $P < 0.01$. Values are means + SE. In the carcinogenesis experiment (*A* and *B*), there were 28 and 27 rats/group in the control group and synbiotic group, respectively; in the ACF or MDF experiments (*C*–*F*), there were 7 rats/group.

Table 1 *Number of ACF and MDF/colon and their multiplicity (ACs/ACF and crypts/MDF) in AOM-treated rats fed a control diet or a diet containing synbiotics*

ACF were evaluated in methylene blue-stained colons, whereas MDF were evaluated in the same methylene blue-stained colons restained with HID-AB. Rats were sacrificed 7 and 15 weeks after AOM.

Weeks after AOM	ACF/colon		AC/ACF		MDF/colon		Crypts/MDF	
	Control	Synbiotic	Control	Synbiotic	Control	Synbiotic	Control	Synbiotic
7	271 ± 15	250 ± 21	2.1 ± 0.1	2.5 ± 0.1[c]	4.2 ± 0.7	5.7 ± 0.6	3.9 ± 0.8	3.9 ± 0.5
15	298 ± 25	322 ± 22[a]	2.6 ± 0.1[b]	3.1 ± 0.1[b,c]	8.2 ± 0.9[b]	3.8 ± 0.9[c]	12.2 ± 2.0[b]	6.4 ± 1.0[a,c]

[a] Significantly different from its respective column value at 7 weeks ($P < 0.05$; means ± SE, $n = 7$).
[b] Significantly different from its respective column value at 7 weeks ($P < 0.01$; means ± SE, $n = 7$).
[c] Significantly different from the value in rats fed the control diet ($P < 0.01$).

and mucinous lesions identified as ACF in HID-AB-stained colons are shown in Fig. 2. Fig. 2*E* shows the same MDF as Fig. 2, *B* and *C*, exhibiting nuclear stratification, anomalies in the structure of the crypts, nuclear crowding, and severe dysplasia in terms of mucin secretion, as expected. In Fig. 2*F*, we present the same lesion at lower magnification, in which the MDF is surrounded by normal crypts. Fig. 2*D* shows the same mucinous lesion identified as ACF as in Fig. 2*A*, in which the production of mucins is still evident.

MDF and mucinous lesions identified as ACF in HID-AB-stained colon with similar multiplicity were harvested from rats sacrificed 7 or 15 weeks after AOM. For each sample, the dysplasia scores attributed to the different parameters were added to calculate a total

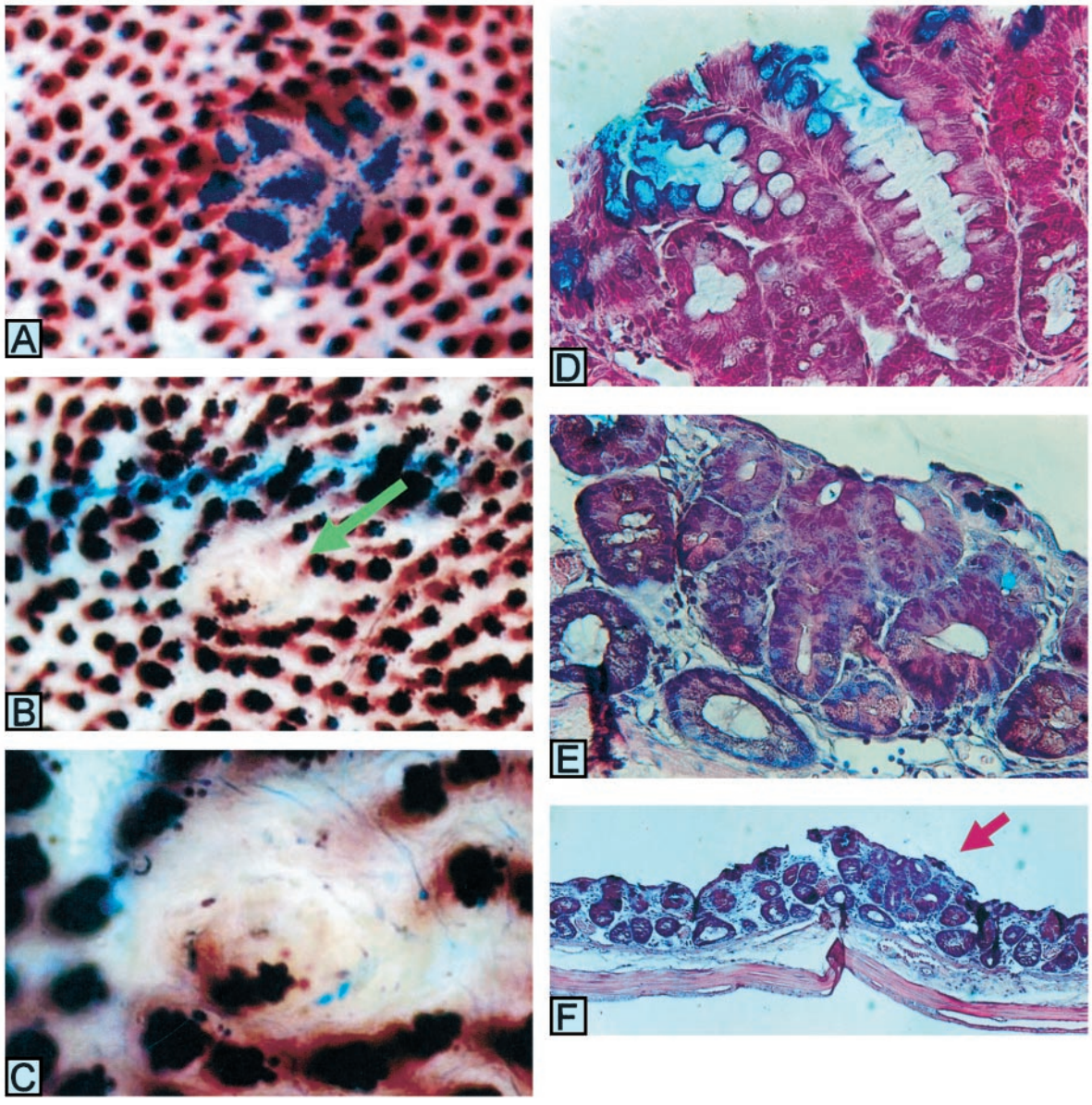

Fig. 2. *A*, topographical identification of a mucinous lesion identified as an ACF formed by 10 crypts in a colon stained with HID-AB (original magnification, ×40). *B*, topographical identification of a MDF (indicated by a *green arrow*) in a colon stained with HID-AB (original magnification, ×40). *C*, the same MDF of *B* at higher magnification (original magnification, ×100). *D*, histological section of the ACF of *A* (original magnification, ×400). *E*, histological section of the MDF of *B* and *C* (original magnification, ×400). *F*, histological section of the same MDF of *E* (indicated by a *purple arrow*) shown at lower magnification (original magnification, ×100).

Table 2 *Grade of histological dysplasia in mucinous lesions identified as ACF in HID-AB-stained colon and MDF harvested 7 and 15 weeks after the first AOM injection*
Values are means ± SD.

	7 weeks		15 weeks	
Dysplasia parameters	ACF (n = 28)	MDF (n = 18)	ACF (n = 28)	MDF (n = 20)
Increase in nuclear:cytoplasmatic ratio	1 ± 0	0.89 ± 0.32	1 ± 0	0.95 ± 0.22
Nuclear stratification	0.53 ± 0.50	0.61 ± 0.50	0.57 ± 0.50	0.8 ± 0.41
Loss of nuclear polarity	0	0.05 ± 0.23	0	0
Irregularity in the nuclear outline	0.04 ± 0.18	0.06 ± 0.23	0	0.2 ± 0.41[a]
Nuclear crowding	0.86 ± 0.36	0.78 ± 0.42	0.75 ± 0.44	0.95 ± 0.22
Number of mitoses	0.11 ± 0.31	0.17 ± 0.38	0.07 ± 0.26	0.25 ± 0.44
Mucin depletion	0.39 ± 0.49	1.5 ± 0.78[a]	0.71 ± 0.59	1.1 ± 0.44[a]
Structural abnormality of the crypts	0.46 ± 0.50	0.72 ± 0.46	0.5 ± 0.51	0.7 ± 0.47
Total score	3.39 ± 1.34	4.78 ± 2.23[a]	3.61 ± 1.61	4.95 ± 1.60[a]

[a] Significantly different when compared with ACF harvested at the same time ($P < 0.01$ by Mann-Whitney U test).

score of dysplasia. The results indicated (Table 2) that both lesions exhibited dysplastic features, but the total dysplasia score considered at both times was significantly higher in MDF than in mucinous lesions identified as ACF in the HID-AB-stained colons ($P < 0.01$, Mann-Whitney t test). The difference in the dysplasia scores of the two lesions was mainly due to severe or mild dysplasia in mucin secretion, which was less marked, as expected, in the mucinous lesions identified as ACF. Fifteen weeks after AOM, the irregularity in the nuclear outline was also more frequent in MDF than in mucinous lesions identified as ACF (Table 2).

Because MDF were identified in colons stained with HID-AB, we did not know whether they would look like ACF or like different lesions in the methylene blue-stained colon. Therefore, we examined methylene blue-stained colons under a microscope (×40 magnification), and we recorded pictures of each visual field. The colon was then restained with HID-AB, and each field in which one MDF was identified was compared with the corresponding field picture stained with methylene blue. This analysis, carried out for 24 MDF, demonstrated that in a methylene blue-stained colon, MDF appeared as very well stained lesions (probably because of a thick layer of epithelial cells). MDF showed a distorted lumen like ACF, but the opening of MDF crypts was not always evident, unlike the ACF, making their identification difficult in a methylene blue-stained colon. The majority of the crypts in MDF (with the exception of 2 MDF of the 24 MDF sampled) were not increased in size, unlike "classical" ACF, which, according to Pretlow and Bird (6), in methylene blue-stained colon have "crypts increased in size, a thickened layer of epithelial cells, increased pericryptal space, irregular lumens and are microscopically elevated."

DISCUSSION

The main finding of this study is the identification of MDF in the unsectioned colon of AOM-treated rats. MDF possess dysplastic features similar to those encountered in colon tumors, and their number and multiplicity are correlated with carcinogenesis in rats fed synbiotics or a control diet. We describe the intestinal cancer induction of this chemopreventive experiment in a recently published paper (16).

Much effort has been dedicated to the identification of preneoplastic lesions in colon carcinogenesis (1, 2, 15). Dysplastic crypts have been described as early lesions in carcinogen-treated rodents and humans at risk (1, 2), and their formation is regarded as a necessary step in the development of colon cancer. However, the identification of dysplastic crypts in experimental animals is difficult and impractical in large-scale studies because it relies on histological procedures. In this context, ACF, first described by Bird in mice treated with AOM (2), may represent perfect end points for short-term carcinogenesis studies. They can be easily scored in the entire unsectioned colon and carry molecular alterations also observed in colon cancer (6). Accordingly, ACF determination has become very popular as a short-term test in experimental carcinogenesis and chemoprevention (11).

In the present study AOM-treated rats were treated with a diet containing synbiotics, which reduce intestinal cancer (16). However, these same dietary components produced opposite results on ACF at earlier time points after AOM induction. ACF multiplicity, a parameter used to predict carcinogenesis (11, 12), even increased in rats treated with synbiotics that inhibit colon cancer. Therefore, ACF and tumor outcome were not correlated in the present study. Such a discrepancy between ACF and carcinogenesis has been described by others (11, 12) and could be partially explained by the fact that not all ACF progress to tumors. In fact, treating rats with a standard dose of AOM (30 mg/kg) induces about 200 ACF/colon after 3 months, but only about 1–2 colon tumors/rat will develop later on. Accordingly, it has been suggested that only a small number of ACF, the dysplastic ones, are true precursors of colorectal cancer (7–10, 19). Similarly, strains of mice genetically susceptible to AOM-induced carcinogenesis have more dysplastic ACF than resistant strains, although ACF develop in both resistant and sensitive strains (14). Recently, Paulsen *et al.* (20) also identified two types of altered crypts in Min/+ mice colon. One of them (ACF_{min}) was dysplastic and characterized by altered growth and defective β-catenin regulation, whereas the other was probably not related to tumorigenesis. All these data suggest that only a fraction of ACF will evolve into cancer.

We report here the identification of an AOM-induced lesion, MDF, characterized by absent or scant mucin production. MDF are easily identified in the unsectioned colons of AOM-treated rats stained with HID-AB, a technique that highlights mucin production (18). The histological characterization of MDF demonstrates dysplastic features characteristic of colon cancer. Similar characteristics, although less marked, were also found in mucinous lesions identified as ACF in HID-AB-stained colons. Dysplastic features have also been reported for ACF in the literature (2–7, 14). MDF already appeared 7 weeks after AOM administration. Their determination 15 weeks after AOM in rats fed synbiotics showed that their number and multiplicity were correlated with carcinogenesis and that their multiplicity increased with time. Moreover, contrary to what is observed with ACF, the number of MDF was in the same order of magnitude as tumors (<10 MDF/colon and 2 tumors/colon, on average).

Our preliminary characterization of MDF does not permit firm conclusions on the relation between MDF and ACF. However, we suggest that MDF are a subgroup of ACF that may predict tumor outcome better than ACF, although this hypothesis requires further validation.

In conclusion, because our data indicate that MDF are dysplastic

lesions that may be precursors of colon cancer, they should be further studied with different carcinogens and dietary treatments.

ACKNOWLEDGMENTS

We thank M. Beni and P. Ceccatelli for expert technical assistance in the management of rats and Mary Forrest for revision of the English usage.

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